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A novel oligoalginate lyase from abalone, *Haliotis discus hannai*, that releases disaccharide from alginate polymer in an exolytic manner

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Abstract—We previously reported the isolation and cDNA cloning of an endolytic alginate lyase, HdAly, from abalone *Haliotis discus hannai* [*Carbohydr. Res.* **2003**, *338*, 2841–2852]. Although HdAly preferentially degraded mannuronate-rich substrates, it was incapable of degrading unsaturated oligomannuronates smaller than tetrasaccharide. In the present study, we used conventional chromatographic techniques to isolate a novel unsaturated-trisaccharide-degrading enzyme, named HdAlex, from the digestive fluid of the abalone. The HdAlex showed a molecular weight of 32,000 on SDS-PAGE and could degrade not only unsaturated trisaccharide but also alginate and mannuronate-rich polymers at an optimal pH and temperature of 7.1 and 42 °C, respectively. Upon digestion of alginate polymer, HdAlex decreased the viscosity of the alginate at a slower rate than did HdAly, producing only unsaturated disaccharide without any intermediate oligosaccharides. These results indicate that HdAlex degrades the alginate polymer in an exolytic manner. Because HdAlex split saturated trisaccharide producing unsaturated disaccharide, we considered that this enzyme cleaved the alginate at the second glycoside linkage from the reducing terminus. The primary structure of HdAlex was deduced with cDNAs amplified from an abalone hepatopancreas cDNA library by the polymerase chain reaction. The translational region of 822 bp in the total 887-bp sequence of HdAlex cDNA encoded an amino-acid sequence of 273 residues. The N-terminal sequence of 16 residues, excluding the initiation methionine, was regarded as the signal peptide of this enzyme. The amino-acid sequence of the remaining 256 residues shared 62–67% identities with those of the polysaccharide lyase family-14 (PL14) enzymes such as HdAly and turban-shell alginate lyase SP2. To our knowledge, HdAlex is the first exolytic oligoalginate lyase belonging to PL14.

Keywords: Alginate lyase; Oligoalginate lyase; *Haliotis discus hannai*; HdAlex; cDNA cloning; Primary structure

1. Introduction

Alginate is an acidic heteropolysaccharide consisting of β-D-mannuronate (M) and α-L-guluronate (G) arranged as a poly(M)-block, a poly(G)-block, and an alternating or random poly(MG)-block.[1–3] Alginate is produced as a structural polysaccharide by brown algae and as an extracellular polysaccharide by certain bacteria.[1–4] The bacterial alginate is in an acetylated form.[3,4] Alginate lyase is an enzyme that catalyzes the degradation of alginate by a β-elimination mechanism, forming 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate at the new non-reducing terminus. This enzyme is found in herbivorous marine mollusks,[5–11] seaweeds,[12,13] marine and soil bacteria,[3,4,14–18] and in *Chlorella* virus.[19] Alginate lyase is primarily classified into poly(M) lyase (EC 4.2.2.3) or poly(G) lyase (EC 4.2.2.11), acting preferentially on the poly(M)-block or poly(G)-block, respectively. Recently, lyases that can degrade both the

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); PCR, polymerase chain reaction.

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poly(M)-block and poly(G)-block have also been isolated.[14,16] The molluscan enzymes are classified as poly(M) lyase.[3,4,6–11,20,21] On the other hand, Henrissat et al. (http://afmb.cnrs-mrs.fr/CAZY/) classified alginate lyases into seven families, that is, polysaccharide lyase families (PLs) -5, -6, -7, -14, -15, -17, and -18, based on their primary structures. Abalone and *Chlorella* virus alginate lyases belong to PL14.

Most alginate lyases are endolytic enzymes that degrade internal glycoside linkages in the alginate chain.[3,4] However, there are also a few alginate lyases possessing exolytic activity. Recently, the exolytic alginate lyase A1-IV that belongs to PL15 was isolated from *Sphingomonas* sp. strain A1 and sequenced.[22,23] A1-IV was originally isolated as the oligoalginate lyase responsible for the complete decomposition of oligoalginates produced by endolytic lyases A1-I, -II, and -III from the same bacterium. In addition, A1-IV exhibited exolytic lyase activity toward alginate polymer, that is, it cleaved the glycoside linkage located in the non-reducing terminus of the alginate chain, producing unsaturated monosaccharide. *Sphingomonas* sp. strain A1 can utilize this monosaccharide as a carbon source.[22–25] The exolytic alginate lyases reported so far all belong to PL15.[22,23,26]

On the other hand, the alginate lyases from mollusks such as abalone,[9–11] turban-shell,[8,21] and *Littorina*[7] are endolytic enzymes that degrade poly(M)-rich substrate producing unsaturated oligosaccharides possessing the unsaturated sugar at the non-reducing terminus, such as unsaturated tri- and disaccharide. The molluscan enzymes are usually incapable of degrading unsaturated oligosaccharides smaller than tetrasaccharides, although the *Haliotis tuberculata* enzyme has been reported to degrade saturated trisaccharide to unsaturated disaccharide and monosaccharide with a low reaction rate.[10] These indicate that mollusks require some other unsaturated-oligoalginate-degrading enzymes for complete decomposition of alginate polymer. At present, there are no reports on the isolation of enzymes that can degrade unsaturated trisaccharide from mollusks, and, of course, no sequence data for this type of enzyme are available.

Previously, we isolated an endolytic alginate lyase, HdAly, from the pacific abalone *H. discus hannai* and cloned its cDNA.[11] HdAly showed high sequence similarity to turban-shell enzyme,[27] and like other molluscan enzymes, produced unsaturated tri- and disaccharides as major end products. On the other hand, we have recently become aware of the presence of an enzyme that can degrade the unsaturated trisaccharide in the digestive fluid of abalone. Here, we report the isolation and characterization of this enzyme. This enzyme could also degrade polymer alginate with an exolytic manner. Further, we cloned cDNA encoding this enzyme and deduced its amino-acid sequence. Accordingly, we found that the sequence showed appreciably high sequence identity relative to the PL14 endolytic alginate lyases. To our knowledge, this is the first report on the isolation and cDNA cloning of the molluscan exolytic alginate lyase that belongs to PL14.

2. Materials and methods

2.1. Materials

The pacific abalone, *H. discus hannai*, (shell size: 6×7 cm) was obtained from the Hokkaido Taisei Aquaculture Center (Taisei, Hokkaido Prefecture, Japan). The endolytic alginate lyase, HdAly, was prepared from the abalone digestive fluid as described previously.[11] TOYOPEARL CM-650M was purchased from Toyo Soda (Tokyo, Japan). Sephacryl S-200 HR, Hydroxyapatite Fast Flow, and Bio-Gel P2 were purchased from Amersham Biosciences AB (Uppsala, Sweden), Wako Pure Chemical Industries (Osaka, Japan), and Bio-Rad Laboratories (Hercules, CA, USA), respectively. The other chemicals used were of reagent grade from Wako Pure Chemical Industries.

2.2. Substrates for alginate lyases

Sodium alginate (*Macrocystis pyrifera* origin; Sigma–Aldrich (St. Louis, MO, USA)) was dissolved in distilled water to make 1% (w/v) and dispersed by heating at 90 °C for 1 h before use. Poly(M)-rich, poly(G)-rich, and random(MG) substrates were prepared from the alginate by the method of Gacesa and Wusteman.[28] The mannuronate content in the original alginate was approximately 60%, whereas the mannuronate contents of the poly(M)-rich and the random(MG) substrates were 86% and 64%, respectively, according to circular dichroism analysis with a J-600 spectropolarimeter (Jasco, Tokyo, Japan). The guluronate content in the poly(G)-rich substrate was 99%. Unsaturated tetra-, tri-, and dimannuronate were prepared from poly(M)-rich substrate by HdAly digestion as follows: poly(M)-rich substrate (0.2 g) was dissolved in 5 mL of 10 mM sodium phosphate (pH 7.0) and digested with 100 U of HdAly at 30 °C for 15 h. The digested material was heated at 95 °C for 5 min and centrifuged at 10,000*g* for 15 min to remove insoluble materials. The supernatant, containing unsaturated oligosaccharides, was lyophilized, dissolved in 1 mL of distilled water, applied to a Bio-Gel P2 column (2.4×95.8 cm) pre-equilibrated with 50 mM sodium phosphate (pH 7.0), and eluted with the same buffer. The oligosaccharides eluted from the column were detected by measuring absorbance at 235 nm, followed by TLC. The unsaturated tetra-, tri-, and disaccharide fractions were pooled, lyophilized, and desalted by gel-filtration through a Bio-Gel P2 column (2.0×47.5 cm) pre-equilibrated with distilled water. Trisaccharide possessing no unsaturated sugar

(term 'saturated trisaccharide' in this paper) was prepared by limited acid hydrolysis of poly(M)-rich substrate according to the method of Muramatsu et al.[29]

2.3. TLC

TLC of oligosaccharides was carried out by the ascending method with Silica Gel-60 TLC plates (E. Merck, Darmstadt, Germany) and a developing solvent of 2:1:1 1-BuOH–HOAc–H_2O. The oligosaccharides developed on the TLC plates were detected by spraying with 10% (v/v) H_2SO_4 in EtOH, followed by heating at 120 °C for 15 min, whereas unsaturated sugars were detected by the thiobarbituric acid method.[30]

2.4. SDS-PAGE

SDS-PAGE was carried out by the method of Porzio and Pearson[31] using 0.1% (w/v) SDS–10% (w/v) polyacrylamide slab gel. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) MeOH–10% (v/v) HOAc, and the background of the gel was destained with 5% (v/v) MeOH–7% (v/v) HOAc.

2.5. Determination of enzyme activity

Alginate lyase activity was determined in 3 mL of reaction mixture containing 0.12% (w/v) substrate, 10 mM sodium phosphate (pH 7.0), and an appropriate amount of enzyme. The progress of the reaction was monitored with a temperature-controlled spectrophotometer (Model U-3010; Hitachi, Tokyo, Japan) by measuring the increase in absorbance at 235 nm due to the formation of double bond between C-4 and C-5 at the new non-reducing terminus by the β-elimination reaction. One unit of lyase activity (U) was defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min. Trisaccharide-degrading activity was assayed by TLC followed by densitometry as follows: 0.1% (w/v) unsaturated trisaccharide in 10 mM sodium phosphate (pH 7.0) was degraded with an appropriate amount of enzyme at 30 °C for various durations, and the reaction was terminated by heating at 95 °C for 3 min. The degradation products (each 2 μL) were subjected to TLC, and the rate of decrease of the trisaccharide was measured with a Shimadzu CS-9000 Chromatoscanner (Shimadzu, Kyoto, Japan). One unit of trisaccharide-degrading activity (U-tri) was defined as the amount of enzyme that degrades 1 μg of trisaccharide for 1 min.

2.6. Purification of trisaccharide-degrading enzyme from abalone

Digestive fluid was collected by aspiration with a plastic syringe from the stomach of the abalone. The fluid from 100 abalones was dialyzed against 10 mM sodium phosphate (pH 7.0) containing 0.1 mM PMSF and 0.2% sodium azide, and centrifuged at 100,000*g* for 1 h. The supernatant was used as crude enzyme. The crude enzyme (10,230 mg total protein) was subjected to ammonium sulfate fractionation, and the fraction precipitated between 40% and 70% saturation (this fraction showed the highest trisaccharide-degrading activity) was collected by centrifugation at 10,000*g* for 15 min. The precipitates were dissolved in and dialyzed against 10 mM Tris–HCl (pH 7.5), and centrifuged at 10,000*g* for 15 min to remove insoluble materials. The supernatant was applied to a column of TOYOPEARL CM-650M (2.5 × 26.5 cm), and the proteins that adsorbed were eluted with a linear gradient of 0–0.3 M NaCl (total volume 600 mL) at a flow rate of 15 mL/h. Trisaccharide-degrading activity was detected in the fractions eluted at around 0.12 M NaCl. The active fractions were pooled and lyophilized. The dried powder was dissolved in 5 mL of cold distilled water and then subjected to gel-filtration through a Sephacryl S-200 HR column (2.0 × 140 cm). The protein showing trisaccharide-degrading activity eluted as a single peak. The elution position indicated that the molecular weight of the enzyme was 30,000–35,000. The active fractions were pooled and applied to a hydroxyapatite column (1.5 × 20 cm) pre-equilibrated with 10 mM Tris–HCl (pH 7.5), and the adsorbed proteins were eluted successively with 10 mM Tris–HCl (pH 7.5), 10 mM

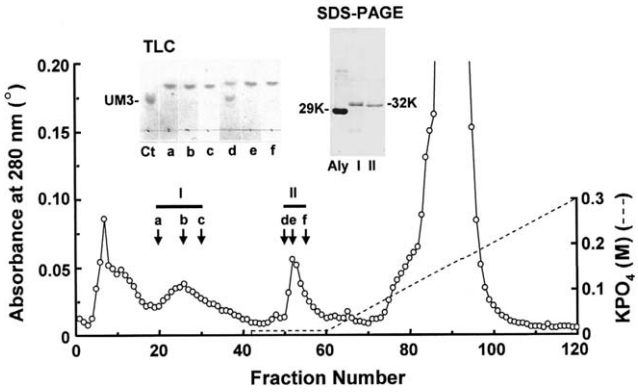

Figure 1. Purification of the trisaccharide-degrading enzyme by hydroxyapatite chromatography. The fractions containing trisaccharide-degrading enzyme in Sephacryl S-200 gel filtration were applied to a hydroxyapatite column (1.5 × 20 cm). The adsorbed proteins were eluted successively with 10 mM Tris–HCl (pH 7.5), 10 mM potassium phosphate (pH 7.0), and a linear gradient of 10–300 mM potassium phosphate (pH 7.0). Each fraction contained 5.0 mL. Ten microliters of each of the fractions indicated by arrows (a–f) was mixed with 10 μL of unsaturated trisaccharide (0.1% (w/v) in water), incubated at 30 °C for 3 h, and analyzed by TLC (inset of the figure). Ct: unsaturated trisaccharide without the enzyme. Fractions indicated by the horizontal solid bars I and II were separately pooled as fractions I and II, respectively. SDS-PAGE of HdAly (Aly) and the fractions I and II are shown in the inset.

potassium phosphate (pH 7.0), and a linear gradient of 10–300 mM potassium phosphate (pH 7.0) (Fig. 1). In this chromatography, the trisaccharide-degrading enzyme was eluted in two peaks, that is, fractions 23–31 eluted with 10 mM Tris–HCl (pH 7.5) and fractions 50–55 eluted with 10 mM potassium phosphate (pH 7.0). Therefore, these two fractions (term 'fractions I and II' in the present paper) were separately pooled and concentrated by ultrafiltration with an Apollo centrifugal concentrator (Orbital Biosciences, Topsfield, MA, USA). Fractions I and II showed a single protein band with an approximate molecular weight of 32,000 on SDS-PAGE (inset of Fig. 1), although the mobility of the fraction II was slightly larger. Since the molecular weight of the enzyme had been estimated to be 30,000–35,000 by the Sephacryl S-200 gel-filtration, both fractions I and II were regarded as monomer enzymes. The molecular weights of HdAlex and the fraction II appeared to be larger than that of HdAly (29,000, inset of Fig. 1). Through the above purification procedure, 0.41 and 0.28 mg of fractions I and II were obtained, respectively (Table 1).

2.7. Determination of protein concentration

Protein concentration was determined by the biuret method[32] or the method of Lowry et al.[33] using bovine serum albumin as a standard protein.

2.8. Determination of amino-acid sequences

The N-terminal amino-acid sequences of the trisaccharide-degrading enzymes, that is, fractions I and II, were determined with a protein sequencer Procise 492HT (Applied Biosystems, Foster City, CA, USA) using specimens that had been transferred to a PVDF membrane after SDS-PAGE. For the analysis of the internal sequences of the fraction I, peptide fragments were prepared as follows: 0.05 mg of fraction I was dissolved in 0.03 mL of 10 mM Tris–HCl (pH 7.5) containing 1% SDS and digested with 1/100 (w/w) of lysylendopeptidase (Wako Pure Chemical Industries) or *Staphylococcus aureus* V8 protease (Wako Pure Chemical Industries) at 37 °C for 30 min. The digests were subjected to SDS-PAGE, electro-blotted to PVDF membrane, and several fragments that were well separated on the membrane were subjected to the sequencer.

2.9. cDNA cloning and nucleotide sequencing

A cDNA library was constructed from an abalone hepatopancreas as described previously.[11] cDNAs encoding the trisaccharide-degrading enzyme were amplified from the library by PCR using degenerated primers synthesized on the basis of partial amino-acid sequences. PCR was performed by using TaKaRa *Taq* DNA polymerase (TaKaRa, Tokyo, Japan) and a PC 700 Program Incubator (ASTEC, Fukuoka, Japan). cDNAs for the 5′- and 3′-terminal regions of mRNA were amplified with 5′- and 3′-full RACE kits (TaKaRa), respectively. The PCR products were cloned with a pCR2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The nucleotide sequence of the cDNA was determined with a BigDye-terminator Cycle sequencing kit (Applied Biosystems) and an ABI 310 Genetic Analyzer (Applied Biosystems). Restriction endonucleases and other DNA-modifying enzymes were purchased from TaKaRa.

2.10. Preparation of recombinant enzyme

The trisaccharide-degrading enzyme was bacterially expressed by using the cloned cDNA and a TOPO expression kit (Invitrogen) along with a host strain *Escherichia coli* Origami B (Novagen, Darmstadt, Germany). The c-myc (-EQKLISEEDL) and octahistidine (-HHHHHHHH) tags were introduced to the C-terminus of the recombinant enzyme to aid detection by Western blotting and purification, respectively. A single colony of transformed Origami B was picked up and cultured overnight in 250 mL of 2 × YT medium at 30 °C. Twelve hours after induction with 0.1 mM isopropyl, 1-thio-β-D-galactopyranoside cells were harvested and sonicated in a buffer containing 10 mM imidazole (pH 8.0), 0.5 M NaCl, 1% Triton X-100, and 0.1 mg/mL lysozyme. After centrifugation at 10,000*g* for 10 min, the supernatant was mixed with Talon Cell-

Table 1. Purification of HdAlex

Steps[a]	Total protein (mg)	Total activity (U-tri)[b]	Specific activity (U-tri mg^{-1})	Yield (%)	Purification (times)
Crude	10,230	64,450	6.30	100	1.00
40–70%	4704	59,021	12.5	91.6	1.98
CM-650M	82.8	16,063	194	25.0	30.8
S-200 HR	14.0	7380	527	11.5	83.7
Fraction I	0.41	1170	2854	1.82	453
Fraction II	0.28	500	1786	0.78	283

[a] Purification steps are abbreviated as follows; Crude: crude enzyme; 40–70%: fraction precipitated between 40% and 70% saturation of ammonium sulfate; CM-650M: active fraction in TOYOPEAL CM-650M chromatography; S-200HR: active fraction in Sephacryl S-200 HR gel filtration; Fractions I and II: the first and second fractions eluted in the hydroxyapatite chromatography.

[b] Trisaccharide-degrading activity (U-tri) was assayed as in the text.

Thru resin (BD Biosciences, Franklin Lakes, NJ) and incubated at 4 °C for 30 min with continuous mixing by rotation. The resin was packed into a column and washed with a buffer containing 30 mM imidazole (pH 8.0) and 0.5 M NaCl. The captured proteins were eluted with a buffer containing 150 mM imidazole (pH 8.0) and 0.5 M NaCl. The fractions containing the recombinant enzyme were pooled, dialyzed against 10 mM sodium phosphate (pH 7.0), and kept on ice until use.

2.11. Western blot analysis for the recombinant enzyme

After SDS-PAGE, the recombinant enzyme was electroblotted to a cellulose nitrate membrane (Toyo Roshi Kaisha, Tokyo, Japan). Anti-c-myc polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as a secondary antibody. Signal detection was performed with a SuperSignal West Pico Chemiluminescent substrate (Pierece Biotechnology, Rockford, IL, USA) and a LAS-1000 Luminoimage Analyzer (Fujifilm, Tokyo, Japan).

3. Results

3.1. Isolation of trisaccharide-degrading enzyme from abalone digestive fluid

The trisaccharide-degrading enzyme was purified as fractions I and II by hydroxyapatite chromatography (Fig. 1). The mobility on SDS-PAGE of the fraction-II enzyme was slightly larger than that of the fraction-I enzyme. However, N-terminal amino-acid sequences of 25 residues of the two enzymes were identical, that is, SIVWTHNEFDPAYFRNGMHSPVTDE. In addition, both of the two enzymes exhibited similar optimum pH's and temperatures (see Fig. 2A and B). These findings suggest that the two are closely related enzymes to each other, for example, they might be produced from the same precursor protein by a different processing of the C-terminus and/or glycosylation. Thus, in the present study, we decided to focus on the fraction I since its yield and specific activity were higher than those of fraction II. Hereafter, we call this enzyme 'HdAlex' after a *Haliotis discus* alginate lyase with an exolytic activity toward alginate polymer that will be shown as follows.

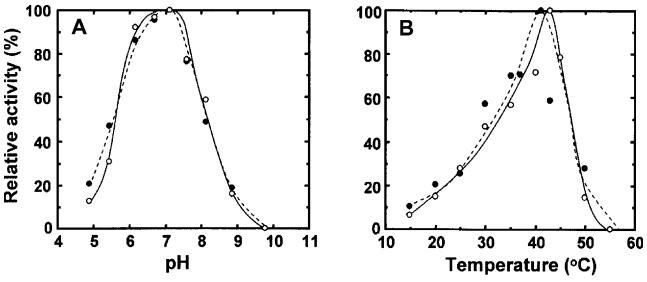

Figure 2. pH and temperature dependences of HdAlex activity. (A) Activities were measured at 30 °C in reaction mixtures containing 0.15% poly(M)-block, 0.21 U/mL of HdAlex (○) or fraction II (●), and 10 mM sodium phosphate with various pHs. (B) Activities were measured at various temperatures in reaction mixtures containing 0.15% poly(M)-block, 0.21 U/mL of HdAlex (○) or fraction II (●), and 10 mM sodium phosphate (pH 7.0).

3.2. Lyase activity of HdAlex toward polymer alginate

To examine whether or not HdAlex could degrade polymer substrates, sodium alginate, poly(M)-rich, random(MG), and poly(G)-rich substrates were allowed to react with HdAlex. HdAlex exhibited the highest activity toward poly(M)-rich substrate, and then sodium alginate and random(MG) substrate in this order, whereas it showed practically no activity toward poly(G)-rich substrate (Table 2). The specific activity of HdAlex was, however, approximately 1/30 of the activity of HdAly. The preference of HdAlex for poly(M)-rich substrate indicates that this enzyme is classified as the mannuronate lyase, as were the previously reported molluscan endolytic alginate lyases.[8,10,11,21] HdAlex as well as the fraction II degraded the poly(M)-rich substrate with an optimum pH and temperature of 7.1 and 42 °C, respectively (Fig. 2A and B).

Then, we investigated the effects of HdAlex on the viscosity of sodium alginate in comparison with those of HdAly. As shown in Figure 3A, HdAly showed a property typical of an endolytic enzyme, that is, it rapidly decreased the viscosity of the alginate in the initial phase of the reaction. On the other hand, HdAlex decreased the viscosity more moderately than did HdAly, even when about 1.5 times as much units of HdAlex as well as that of HdAly were added to the reaction mixture. These results strongly suggested that HdAlex degraded the alginate in an exolytic manner, unlike HdAly. In addition, it should be noted that the rate of increase in absorbance at 235 nm by HdAlex

Table 2. Substrate specificity of HdAlex

Enzyme	Substrate	Specific activity ($U\ mg^{-1}$)[a]	Relative activity (%)
HdAlex	Sodium alginate	129	100
	Poly(M)-rich	684	530
	Poly(G)-rich	ND[b]	—
	Random(MG)	57.9	45
HdAly	Sodium alginate	3831	100
	Poly(M)-rich	17,927	470
	Poly(G)-rich	ND[b]	—

[a] One unit of lyase activity is defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min.
[b] Not detectable.

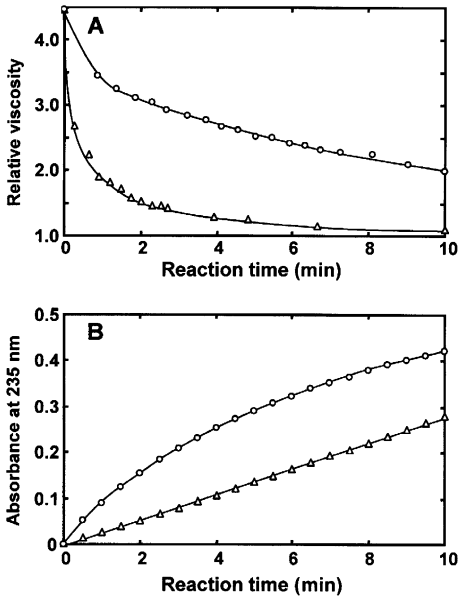

Figure 3. Changes in the viscosity of alginate polymer and in absorbance at 235 nm by the digestion with either HdAlex or HdAly. (A) Decrease in viscosity of sodium alginate during digestion with HdAlex (○) or HdAly (△). The reaction was carried out at 30 °C in an Ostwald-type viscometer, in a mixture containing 0.12% (w/v) sodium alginate, 10 mM sodium phosphate (pH 7.0), and 3.93 U/mL of HdAlex or 2.78 U/mL of HdAly. The flow time of the buffer was 25 s. (B) Changes in absorbance at 235 nm by digestion with HdAlex (○) or HdAly (△). Digestion was carried out at 30 °C using the same mixture as in (A).

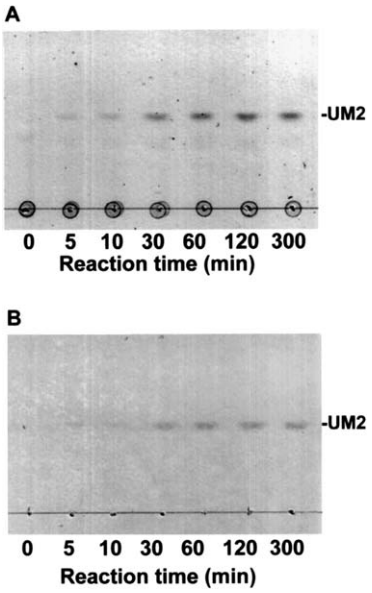

Figure 4. Degradation of poly(M)-rich substrate by HdAlex. The reaction mixture containing 0.1% (w/v) poly(M)-rich substrate, 10 mM sodium phosphate (pH 7.0), and 4.4 U/mL HdAlex was incubated at 30 °C for 5–300 min. Aliquots of the mixture (2 μL) were subjected to TLC. (A) Oligoalginates detected with 10% (v/v) sulfuric acid. (B) Unsaturated oligoalginates detected by the thiobarbituric acid method.[30] UM2, unsaturated disaccharide.

gradually declined as the reaction proceeded (Fig. 3B). We consider that this decline in the reaction rate of HdAlex is ascribable to exhaustion of the poly(M)-rich and random(MG) regions in the substrate alginate, followed by the exposure of poly(G)-rich regions in the alginate termini as a result of the exolytic digestion by HdAlex.

3.3. Degradation of poly(M)-rich substrate and saturated trisaccharide by HdAlex

As described above, HdAlex was considered as an exolytic mannuronate lyase. Then, we analyzed the degradation products of poly(M)-rich substrate produced by HdAlex. TLC analysis indicated that HdAlex released only disaccharide from poly(M)-rich substrate, with no intermediate oligosaccharides (Fig. 4A). Staining with thiobarbituric acid revealed that the disaccharide thus released contained unsaturated sugar, whereas the residual polymer products remaining at the starting point of TLC contained no unsaturated sugar (Fig. 4B). These results indicated that the HdAlex acted as an exolytic lyase, releasing unsaturated disaccharide directly from the poly(M)-rich substrate.

We then investigated, which terminus of the substrate, reducing or non-reducing, was degraded by HdAlex. Namely, we degraded saturated trisaccharide with HdAlex, and analyzed, which product, disaccharide or monosaccharide, is the unsaturated form. As a result, the disaccharide was found to be produced as the unsaturated form although the monosaccharide was hardly detected by the thiobarbituric assay (Fig. 5A and B). Since all the alginate lyases so far reported are known to form a double bond between C-4 and C-5 at the non-reducing terminus of the split site,[2–4] we can conclude that HdAlex split the second glycosyl linkage from the reducing terminus of the trisaccharide (Fig. 5C). Taking together the results obtained with the poly(M)-rich substrate and the saturated trisaccharide, HdAlex was regarded as an enzyme that exolytically degrades the reducing terminus of alginate (poly(M)-block) forming unsaturated disaccharide. The cleavage site of saturated trisaccharide by HdAlex seemed to be identical to that reported on *H. tuberculata* endolytic enzyme.[10] However, the *H. tuberculata* enzyme could not degrade unsaturated trisaccharide unlike HdAlex. On the other hand, HdAlex was considered to be incapable of degrading unsaturated disaccharide because the disaccharide produced from either the poly(M)-rich substrate or trisaccharide were not further degraded any more (see Figs. 4 and 5). Furthermore, HdAlex could not degrade the isolated unsaturated disaccharide, and it more slowly degraded the unsaturated tetrasaccharide than unsaturated trisaccharide (Fig. 6). These results suggest that the preferable substrate of HdAlex in the abalone digestive fluid is the unsaturated trisaccharide produced by HdAly.

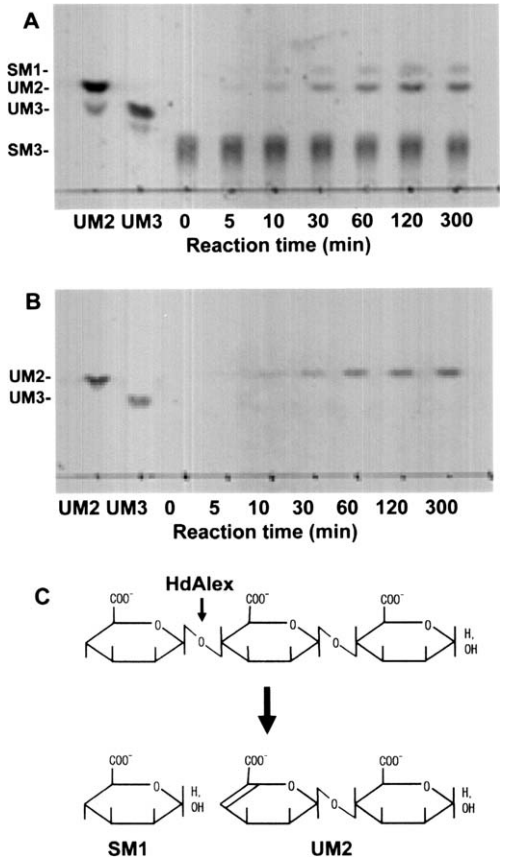

Figure 5. Degradation of saturated trisaccharide by HdAlex. Saturated trisaccharide (SM3) prepared by limited acid hydrolysis was degraded by 4.4 U/mL HdAlex under the same conditions as in Figure 4. Aliquots of the reaction mixture (2 μL) were subjected to TLC. (A) Oligoalginates were detected with 10% sulfuric acid. (B) Unsaturated oligoalginates were detected with thiobarbituric acid. SM1, saturated monosaccharide; UM2, unsaturated disaccharide; UM3, unsaturated trisaccharide; SM3, saturated trisaccharide. (C) Schematic diagram for the action of HdAlex on saturated trisaccharide.

3.4. Primary structure of HdAlex

As described above, the amino-acid sequence of the N-terminal 25 residues of HdAlex was determined as SIVWTHNEFDPAYFRNGMHSPVTDE. This sequence showed approximately 52% identity with that of HdAly. The sequences of lysylendopeptidase fragments of HdAlex, L1 (VQFFATPTKPRVAMTLSYDV) and L2 (GFSFGRG), and a V8 protease fragment, V1 (KGSYSHHGPNSKVQF), also showed 70–73% identity with the corresponding sequences of HdAly. Accordingly, HdAlex was considered to possess a similar primary structure to HdAly. Therefore, we deduced the complete primary structure of HdAlex by the cDNA method as follows.

Degenerated primers for PCR were synthesized on the basis of partial amino-acid sequences of HdAlex. Namely, a forward primer AlexFw (5′-TGGACN-CAYAAYGARTTYGA-3′) was synthesized from the WTHNEFD sequence in the N-terminus of HdAlex, whereas a reverse primer AlexRv (5′-GTTGTTACTGGCGTACACCAC-3′) was from the TKPRVAM sequence of the lysylendopeptidase fragment L1 that deviated appreciably from the corresponding sequence of HdAly. By using the AlexFw–AlexRv primer pair, PCR-DNA of 219 bp, which encodes an amino-acid sequence of 73 residues of HdAlex, was amplified. Then, a specific primer 3F (5′-ACC-ATGGGCCAAACAGCAAGG-3′) was synthesized from the sequence of A_{161}–G_{182} in the PCR-DNA, and 3′-RACE PCR was performed with this primer. This PCR gave 3′RACE-DNA consisting of 688 bp. We also synthesized specific primers 5R2 (5′-CCG-TATAGGCGGCAAACTTCCTGG-3′) from the sequences of C_{54}–G_{77} in the PCR-DNA and amplified 5′RACE-DNA consisting of 181 bp. By overlapping the nucleotide sequences of the 5′RACE-DNA, PCR-DNA, and 3′RACE-DNA in this order, a nucleotide sequence of 949 bp in total was determined. The reliability of this sequence was confirmed with Full-DNA consisting of 887 bp that was newly amplified with the specific primers Full5Fw (5′-CCAGACAGGAATGAA GATGTGGAC-3′) and Full3Rv (5′-ACAACCACA-CAATGGTGAAGATCA-3′). The nucleotide and the deduced amino-acid sequence data are available from the DNA Data Bank of Japan with an accession number AB234872.

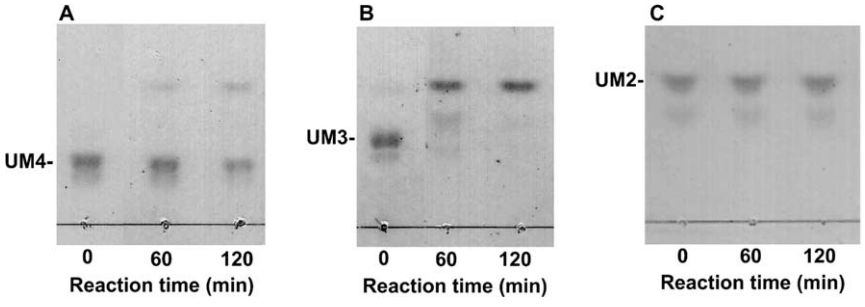

Figure 6. Degradation of unsaturated oligosaccharides by HdAlex. Unsaturated oligosaccharides were incubated with 2.6 U/mL HdAlex in the mixture containing 0.1% of each substrate, 10 mM sodium phosphate (pH 7.0), at 30 °C for 60 and 120 min. (A)–(C) Unsaturated tetra- (UM4), tri- (UM3), and disaccharide (UM2), respectively. The reaction products were subjected to TLC and stained with sulfuric acid.

In the nucleotide sequence for HdAlex cDNA, the translational initiation codon ATG was found in A_{45}–G_{47} and termination codon TGA was found in T_{864}–A_{866}, whereas a putative polyadenylation signal sequence, TATTTAAA, was found in residues T_{916}–A_{923}, and a poly(A) sequence of 15 residues was found in the 3′-terminus. The sequence of the translational region consisting of 822 nucleotides encoded an amino-acid sequence of 273 residues. All partial amino-acid sequences determined from the HdAlex protein can be seen in the deduced sequence; however, the 17 N-terminal residues, including the initiation methionine, are not seen in the N-terminal of HdAlex protein. Since this N-terminal sequence showed a quite high similarity to the consensus sequence of eukaryote secretary proteins,[34] we considered it to be the signal peptide region of HdAlex. Consequently, we concluded that the mature HdAlex consists of 256 residues. The amino-acid sequence of HdAlex shared 67.2%, 61.8%, and 34.1% identities with abalone HdAly,[11] turban-shell SP2,[27] and *Chlorella* virus CL2,[19] respectively (Fig. 7). This indicates that HdAlex also belongs to PL14 like those of PL14 alginate lyases.

3.5. Trisaccharide-degrading activity of recombinant HdAlex

To confirm that HdAlex cDNA actually encodes the HdAlex protein, we prepared recombinant HdAlex and determined its trisaccharide-degrading activity. The cDNA fragment encoding the mature HdAlex protein, that is, the region corresponding to amino-acid residues 18–273 (see Fig. 7), was subcloned to an expression vector pET101 by using a Champion pET directional TOPO expression kit (Invitrogen) and expressed in Origami B (Novagen). The recombinant HdAlex was fairly well expressed in the soluble fraction and successfully purified by affinity chromatography with Talon CellThru resin (Fig. 8A and B). The purified recombinant HdAlex could degrade unsaturated trisac-

```
HdAlex-I 18  ---SIVWTHN EFDPAYFRNG MH-------- ---------- ---------- ----------    36
HdAly    18  ---AVLWTHK EFDPANYRNG MH-------- ---------- ---------- ----------    36
SP2       1  ---TLLWTHK EFDPNNYRDG MH-------- ---------- ---------- ----------    19
CL2       1  MNGNDNWDNV VKDYNNLRKN GHDEQETISI IRRKYTDIGP VNQKRLEEQY EKTKPSPKPA    60
                    * :    *    *..  *

HdAlex-I     ---------- ---------- ---------- ----SPVTDE DVNGSATVVP D---------    53
HdAly        ---------- ---------- ---------- ----ALTSND YDHGSGSVVT D---------    53
SP2          ---------- ---------- ---------- ----ALTSND YDHGSGKVVT D---------    36
CL2          PKSAPKPAPK PAPKPAPKTA PKPPPATKNT NVISTLDLNL LTKGGGSWNV DGVNMKKSAV   120
                                                  :   :    :*...    *

HdAlex-I     PNGGSNLVLK VFYEKGSYSH HGPNSK-VQF FATPT-KPRV AMTLSYDVRF DPNFDFRIGG   111
HdAly        PDGGSNHVLR VWYEKGRYSS HGPNEG-VQF FATPT-QDHS IMTFSYDVYF DKNFDFRRGG   111
SP2          PDGGSNHVLR VWYEKGRWSS HGPNEG-VQF FATPT-QDHS VMTFSYDLYL SHDFDFRRGG    94
CL2          TTFDGKRVVK AVYDKNSGTS ANPGVGGFSF SAVPDGLNKN AITFAWEVFY PKGFDFARGG   180
             .  ..: *:: . *:*.  :   .*.   ..*  *.*    :   :*:::::     .***  **

HdAlex-I     KLPGLYGGLV NCSGGRHSDD CFSTRFMWRD NGDGEVYGYV PDQSHQLPGF CTKNICDPVK   171
HdAly        KLPGLFGGWT NCSGGRHSDN CFSTRFMWRA DGDGEVYGYI QNKDHQIDGF CDHVVCNSIK   171
SP2          KLPGLYGGWT NCSGGRHSDN CFSTRFMWRK DGDGEVYAYI PDYHHQVSGF CDHNVCNSVK   154
CL2          KHGGTFIGHG AASGYRHSKT GASNRIMWQE KGGVIDYIYP PSDLKQKIPG LDP--EGQGI   238
             *  * : *    .** ***.   *.*:**:  .*.   * *   .  :*          .

HdAlex-I     GFSFGRGSWR FQRGVWQTIA QSIKLNTPG- ---STDGAIK VVYNGKVVYA SNNLALRSQS   227
HdAly        GYSMGRGKWR FQRGKWQNIA QSVKLNTPG- ---KTDGSIK VWYNGKLVFT IDQLNIRAKA   227
SP2          GYSLGRGKWK FERGKWQNIA QHVHLNTPG- ---KTDGSIK VWHNGKLVYT IDQLNIVSKA   210
CL2          GFFQDDFKNA LKYDVWNRIE IGTKMNTFKN GIPQLDGESY VIVNGKKEVL KG-INWSRSP   297
             *:  .  .   :: . *: *    ::**       . **    *  ***      . :    ..

HdAlex-I     DVNIDGIFFS TFFGGSYANW APTRDCYTWF KNFAISFDTG PEVAVG---- -------      273
HdAly        SVDLDGIFFS TFFGGHDSTW APTHDCYSYF KNFVLSTDSG HPTIIG---- -------      273
SP2          SVDIDGIFFS TFFGGSDSSW APTHDCYSYF KNFALSTDSS HPTIL----- -------      253
CL2          ELLISRFDWN TFFGGPLP-- -SPKNQVAYF TNFQMKKYE- ---------- -------      333
             .: :. : :. *****  .    ..::  ::* .**  :.
```

Figure 7. Comparison of amino-acid sequences of HdAlex and PL-14 alginate lyases. The amino-acid sequence of HdAlex was aligned with those of HdAly,[10] turban-shell SP2,[26] and *Chlorella* virus CL2.[18] Identical, highly conservative, and conservative residues among sequences are indicated by asterisk (*), colon (:), dot (.), respectively. Regions showing relatively high similarity among the sequences are boxed.

charide to disaccharide and monosaccharide (scarcely detected by TLC), as did native HdAlex (Fig. 8C). Thus, the cloned cDNA was confirmed to be the cDNA encoding the trisaccharide-degrading enzyme, HdAlex.

4. Discussion

4.1. Isolation of exolytic alginate lyase HdAlex from abalone digestive fluid

It is widely accepted that herbivorous marine mollusks possess alginate lyases as digestive enzymes.[3–11] In a previous paper, we reported on the isolation, biochemical properties, and primary structure of HdAly, an endolytic alginate lyase from abalone *H. discus hannai*.[11] Similar to other known molluscan alginate lyases, HdAly produced unsaturated tri- and disaccharides as major end products from poly(M)-rich substrate, and could not degrade the unsaturated oligosaccharides smaller than a tetrasaccharide. Accordingly, we considered that abalone needs other oligoalginate-degrading enzymes distinct from HdAly for complete depolymerization of alginate. In the present study, we successfully isolated an enzyme that could degrade the unsaturated trisaccharide and named it as HdAlex, since the enzyme showed an exolytic activity toward polymer substrate. HdAlex produced unsaturated disaccharide from poly(M)-rich substrate without forming any intermediate oligosaccharides, and split saturated trisaccharide producing unsaturated disaccharide and monosaccharide. Accordingly, the cleavage site of the polymer substrate by HdAly was regarded as the second glycoside linkage from the reducing terminus. To our knowledge, HdAlex is the first molluscan oligoalginate lyase possessing exolytic activity toward polymer alginate. Previously, an exolytic alginate lyase, A1-IV, was isolated from *Sphingomonas* sp. strain A1.[22,23] A1-IV cleaves the first glycoside linkage from the non-reducing-terminus producing unsaturated monosaccharide. Thus, HdAlex and A1-IV are the exolytic alginate lyases that act on the opposite terminus of alginate chain producing different products. HdAlex could not degrade unsaturated disaccharide, whereas it degraded unsaturated tetrasaccharide with a slower rate than unsaturated trisaccharide (Fig. 6). These results suggest that the most preferable substrate of HdAlex in abalone digestive fluid is unsaturated trisaccharide produced by HdAly. It is noteworthy that the cleavage site of saturated trisaccharide by HdAlex is the same as that by an endolytic *H. tuberculata* enzyme,[10] which cleaved the second glycosylic linkage from the reducing terminus. Although *H. tuberculata* enzyme could not degrade unsaturated trisaccharide, the molluscan lyases may potentially degrade the saturated trisaccharide.

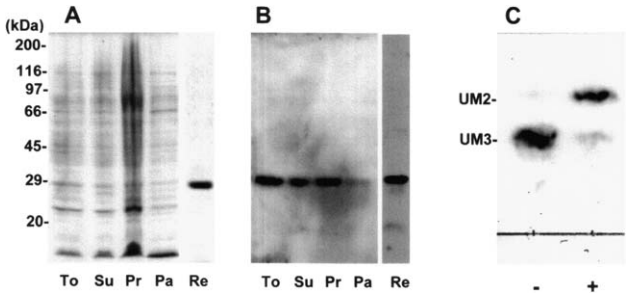

Figure 8. Preparation of recombinant HdAlex and its trisaccharide-degrading activity. (A) SDS-PAGE of samples from various purification steps. To: Total cell lysate after IPTG induction; Su and Pr: supernatant and precipitates, respectively, of cell homogenates after centrifugation at 10,000*g* for 10 min; Pa: pass fraction of the supernatant through Talon CellThru resin; Re: recombinant HdAlex eluted from the resin with 150 mM imidazole (pH 8.0) and 0.5 M NaCl. (B) Western blot analysis of each sample with anti-c-myc antibody. (C) Degradation of trisaccharide by the recombinant HdAlex. Unsaturated trisaccharide (UM3) was degraded by the recombinant HdAlex in a reaction mixture containing 0.1% (w/v) UM3, 10 mM sodium phosphate (pH 7.0), and 3 U/mL recombinant HdAlex. TLC was carried out as in Figure 1. Plus (+) and minus (−) indicate with and without recombinant HdAlex. The oligosaccharides were detected with sulfuric acid.

4.2. Primary structure of HdAlex

We cloned the cDNAs encoding HdAlex and compared the deduced amino-acid sequence with the sequences of other PL14 alginate lyases (Fig. 7). The amino-acid sequence of HdAlex shared 67.2%, 61.8%, and 34.1% identities with abalone HdAly,[11] turban-shell SP2,[27] and *Chlorella* virus CL2,[19] respectively. This indicates that HdAlex also belongs to PL14. On the other hand, it shared practically no identity with the other PL families comprising bacterial alginate lyases.[4,15–18] Up to now, there is no report on exolytic alginate lyase belonging to PL14. Thus, HdAlex is regarded as the first exolytic PL14 enzyme.

Since the primary structure of HdAlex shares 67.2% identity with that of HdAly, a comparison of the two sequences may provide useful information about differences between the exolytic and endolytic mechanisms of alginate lyases. As shown in Figure 7, there are several regions well conserved between HdAlex and other PL14 endolytic enzymes, that is, Val_{61}–Lys_{68}, Met_{93}–Val_{99}, Phe_{105}–Lys_{112}, Ser_{124}–Ser_{129}, Ser_{134}–Arg_{140}, Tyr_{148}–Gln_{157}, Lys_{195}–Thr_{198}, Asn_{211}–Lys_{213}, Thr_{238}–Gly_{242}, and Thr_{255}–Phe_{260} (residue numbers are those of HdAlex). Cysteine pairs Cys_{106}–Cys_{115} and Cys_{145}–Cys_{150}, which have been suggested to form disulfide linkages in turban-shell SP2,[27] are also conserved in HdAlex as Cys_{123}–Cys_{132} and Cys_{162}–Cys_{167} (residue numbers are those including signal peptide). In addition, an Asn in a putative carbohydrate-chain-anchoring motif, Asn_{122}–Cys–Ser_{124} shown in SP2,[27] is also conserved. These conservations seem to be fundamentally important for catalytic actions of both endolytic and

exolytic enzymes. On the other hand, Pro residues specific to HdAlex sequence occur at positions 38, 52, 89, 103, 159, 169, and 268. In addition, there are numerous substitutions of hydrophobic and polar residues between HdAly and HdAlex, for example, Leu versus Phe, Phe versus Trp, His versus Ser, Arg versus Tyr, etc. In order to reveal the structural regions involved in the endolytic and exolytic actions of abalone alginate lyases, we are now attempting to construct various kinds of site-directed and chimeric mutants for HdAly and HdAlex.

The molecular weight of the mature HdAlex calculated from the deduced amino-acid sequence was 28318. This value is considerably different from that estimated by SDS-PAGE and Sephacryl S-200 gel filtration. We now consider that this difference is ascribable to the glycosylation of HdAlex. Namely, in the case of turbanshell SP2,[27] a carbohydrate chain consisting of one residue of *N*-acetylglucosamine, three residues of fucose, and one residue of mannose has been reported to anchor in Asn_{105}. As described above, HdAlex also conserved the carbohydrate-chain-anchoring motif. The structure and size of the putative carbohydrate chain in HdAlex is now under investigation.

4.3. Roles of HdAlex as a digestive enzyme

In certain bacteria, α-keto acid converted from unsaturated uronic-acid monosaccharide can be metabolized by the glycolysis system. We consider that unsaturated monosaccharide, along with unsaturated disaccharide, can be produced by the actions of HdAly and HdAlex. To clarify whether or not the glycolysis system of abalone can utilize the unsaturated monosaccharide, investigation of the pathway concerned with the decomposition of disaccharides to monosaccharide is also important. Recently, it was reported that some intestinal bacteria of abalone metabolize oligoalginate into organic acids such as lactic acid and acetic acid.[35] These organic acids might be utilized by abalone. To reveal the overall utilization pathway of alginate as a carbon source in abalone, we are now searching for enzymes that can degrade unsaturated disaccharide, which is not degraded even by HdAlex, in the abalone digestive organ. Studies on the disaccharide-degrading enzymes will also provide useful information for us to understand the functional characteristics of molluscan alginate lyases.

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References

1. Haug, A.; Larsen, B.; Smidsrød, O. *Acta Chem. Scand.* **1967**, *21*, 691–704.
2. Gacesa, P. *Carbohydr. Polym.* **1988**, *8*, 161–182.
3. Gacesa, P. *Int. J. Biochem.* **1992**, *24*, 545–552.
4. Wong, T. Y.; Preston, L. A.; Schiller, N. L. *Annu. Rev. Microbiol.* **2000**, *54*, 289–340.
5. Nakada, H. I.; Sweeny, P. C. *J. Biol. Chem.* **1967**, *242*, 845–851.
6. Nishizawa, K.; Fujibayashi, S.; Kashiwabara, Y. *J. Biochem. (Tokyo)* **1968**, *64*, 25–37.
7. Elyakova, L. A.; Favorov, V. V. *Biochim. Biophys. Acta* **1974**, *358*, 341–354.
8. Muramatsu, T.; Egawa, K. *Agric. Biol. Chem.* **1977**, *46*, 883–889.
9. Boyen, C.; Kloareg, B.; Polne-Fuller, M.; Gibor, A. *Phycologia* **1990**, *29*, 173–181.
10. Heyraud, A.; Colin-Morel, P.; Girond, S.; Richard, C.; Kloareg, B. *Carbohydr. Res.* **1996**, *291*, 115–126.
11. Shimizu, E.; Ojima, T.; Nishita, K. *Carbohydr. Res.* **2003**, *338*, 2841–2852.
12. Madgwick, J.; Haug, A.; Larsen, B. *Acta Chem. Scand.* **1973**, *27*, 711–712.
13. Watanabe, T.; Nishizawa, K. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 243–249.
14. Sawabe, T.; Ohtsuka, M.; Ezura, Y. *Carbohydr. Res.* **1997**, *304*, 69–76.
15. Sugimura, I.; Sawabe, T.; Ezura, Y. *Mar. Biotechnol.* **2000**, *2*, 65–73.
16. Iwamoto, Y.; Araki, R.; Iriyama, K.; Oda, T.; Fukuda, H.; Hayashida, S.; Muramatsu, T. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 133–142.
17. Murata, K.; Inose, T.; Hisano, T.; Abe, S.; Yonemoto, Y.; Yamashita, T.; Takagi, M.; Sakaguchi, K.; Kimura, A.; Imanaka, T. *J. Ferment. Bioeng.* **1993**, *76*, 427–437.
18. Chavagnat, F.; Duez, C.; Guinand, M.; Potin, P.; Barbeyron, T.; Henrissat, B.; Wallach, J.; Ghuysen, J. M. *Biochem. J.* **1996**, *319*, 575–583.
19. Suda, K.; Tanji, Y.; Hori, K.; Unno, H. *FEMS Microbiol. Lett.* **1999**, *180*, 45–53.
20. Haugen, F.; Kortner, F.; Larsen, B. *Carbohydr. Res.* **1990**, *198*, 101–109.
21. Muramatsu, T.; Egawa, K. *Agric. Biol. Chem.* **1980**, *44*, 2587–2594.
22. Hashimoto, W.; Miyake, O.; Momma, K.; Kawai, S.; Murata, K. *J. Bacteriol.* **2000**, *182*, 4572–4577.
23. Miyake, O.; Hashimoto, W.; Murata, K. *Protein Exp. Purif.* **2003**, *29*, 33–41.
24. Yamasaki, M.; Moriwaki, S.; Miyake, O.; Hashimoto, W.; Murata, K.; Mikami, B. *J. Biol. Chem.* **2004**, *279*, 31863–31872.
25. Yoon, H. J.; Mikami, B.; Hashimoto, W.; Murata, K. *J. Mol. Biol.* **1999**, *290*, 505–514.
26. Hashimoto, W.; Miyake, O.; Ochiai, A.; Murata, K. *J. Biosci. Bioeng.* **2005**, *99*, 48–54.
27. Muramatsu, T.; Komori, K.; Sakurai, N.; Yamada, K.; Awasaki, Y.; Fukuda, K.; Oda, T. *J. Protein Chem.* **1996**, *15*, 709–719.
28. Gacesa, P.; Wusteman, F. S. *Appl. Environ. Microbiol.* **1990**, *56*, 2265–2267.
29. Muramatsu, T.; Yamada, K.; Date, M.; Yoshioka, S. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1990–1994.
30. Linning, M. C.; Cohen, S. S. *J. Biol. Chem.* **1951**, *189*, 109–114.

31. Porzio, M. A.; Pearson, A. M. *Biochim. Biophys. Acta* **1977**, *490*, 27–34.
32. Gornall, A. G.; Bardawill, C. J.; David, M. M. *J. Biol. Chem.* **1949**, *177*, 751–766.
33. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
34. von Heijne, G. *Nucleic Acids Res.* **1986**, *14*, 4683–4690.
35. Sawabe, T.; Setoguchi, N.; Inoue, S.; Tanaka, T.; Ootsubo, M.; Yoshimizu, M.; Ezura, Y. *Aquaculture* **2003**, *219*, 671–679.